REMARKS

Claims 1-53 are pending in this application. Claims 29-53 are withdrawn. Claims 1-4, 9-13, 16-19, and 22-26 are amended. Support for the amendments to these claims can be found, for example, in old claims 1-4, 9-13, 16-19, 22-26, and in the specification (see pg. 8, lines 14-17). Reconsideration is respectfully requested in view of the following remarks.

I. Claim Rejection Under 35 U.S.C. 112, First Paragraph:

The Examiner rejected claims 1-28 under 35 U.S.C. 112, first paragraph, as being allegedly non-enabling for the claimed invention. Applicant traverses-in-part and has amended-in-part the instant claims to address this rejection.

The Examiner provides an analysis of the Wands factors for the proposition that it provides factors for analysis in determining when a disclosure for a patent application requires undue experimentation. The Examiner thus concludes that the factors presented provide that "undue experimentation would have been required at the time of the effective filing date of the instant application for one of ordinary skill in the art to reproducibly practice the full scope of the present invention, as claimed." See Office Action, pg. 5.

Applicants wish to point out that the amount of experimentation is only one of a lengthy list of factors that the Federal Circuit has suggested in determining whether the scope of claims in a patent application or patent are enabled by the disclosure. The list of the factors to be considered are:

(A) The breadth of the claims;

(B) The nature of the invention;

(C) The state of the prior art;

(D) The level of one of ordinary skill;

(E) The level of predictability in the art;

(F) The amount of direction provided by the inventor;

(G) The existence of working examples; and

(H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

Furthermore, the court in Wands states:

> It is improper to conclude that a disclosure is not enabling based on an analysis of only one of the above factors while ignoring one or more of the others. The examiner's analysis must consider all the evidence related to each of these factors, and any conclusion of nonenablement must be based on the evidence as a whole. *In re Wands*, 858 F.2d 731 at 737,740 (Fed. Cir. 1988).

In regards to the amount of experimentation needed to support an undue experimentation argument, the test itself "is not merely quantitative, since a considerable amount of experimentation is permissible if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the claimed invention." *Id.* at 736-740. Applicants submit that the specification provides sufficient guidance to enable the amended claims without undue experimentation in the instant application.

In addition, Applicants respectfully disagree with the Examiner's contention regarding botulinum toxin prior art. The Examiner points to Boroff et al. (pg. 1-62, in "Microbial Toxins" S Kadis, T. Montie and S. Ajl, eds., Academic Press, 1971) for the assertion that the stability of the claimed type B toxin liquid formulation within the full concentration range as recited in the instant claims. The Examiner claims that this is important because the art allegedly reports lack of clarity and contradictions on the subject of stability of botulinum toxins as related to various factors. Applicants submit that the Boroff et al. article does not represent the state of the art at the time of filing of the instant application.

The Boroff article, published in 1971, is a review of the state of the art in the 50's and 60's. The article presents work done on primarily partially purified extracts of botulinum toxin preparations (see pgs. 9-15). In contrast, the present application provides detailed guidance with respect to the isolation and purification of botulinum toxin complexes. The present application comprehends the need for purification from cellular proteases and contaminants, in contrast to the work cited in the Boroff article, in order to maintain the stability of the liquid formulation at the temperatures cited. Therefore, the Boroff article is not relevant to the state of the prior art at the time of filing, and does not support the Examiner's contention that there are contradictions on the subject of stability of botulinum toxins as related to various factors.

In regards to the Examiner's reliance on Gartlan et al. (Otolar-Head & Neck Surg. 108:135-140 (1993)) regarding the stability of botulinum toxin at pH 6.2 phosphate buffer, Applicants respectfully point out that the reference does not apply to the instant claims. The Examiner states that Gartlan supports art describing botulinum toxin solution stability at room temperature in phosphate buffer of pH 6.2 for only a few days. Applicants respectfully disagree with the Examiner that Gartlan supports this interpretation. As support, Applicants point to the article referenced by Gartlan, specifically to the work of Schantz and Kautter ("Standardized assay for clostridium botulinum toxins," J. Assoc. Off. Anal. Chem. 61:96-9 (1978), attached herein) which describe experiments in *freeze-thawing* of botulinum toxin solutions. As stated in the Gartlan article at p. 139, 2nd full paragraph, "Schantz and Kautter found that botulinum toxin can be *frozen and thawed* without appreciable loss of toxicity when buffered in a sodium phosphate solution of pH 6.2" (emphasis added). That Gartlan was speaking to results from freeze-thaw experiments is supported in the Schantz and Kautter article on p. 98, 2nd col., 1st full paragraph. In describing the experiments on sodium phosphate buffer, Schantz states "In this buffer the solution can be *frozen and thawed* without appreciable loss of toxicity." The instant application does not involve freeze-thawing of botulinum toxin solutions, a procedure that is known to those of ordinary skill in the art to be harmful to protein complexes. Therefore, Gartlan is not applicable to the instant claims.

Similarly, the Examiner's reliance on Schantz & Johnson (In "Therapy with Botulinum Toxin" Jankovic et al. eds., Marcel Dekker, Inc., New York, 41-51 (1994)) is also not applicable to the instant claims. The Examiner points to Schantz & Johnson for the contention that "purified neurotoxins, i.e., neurotoxins separated from the protective nontoxic proteins, exhibited poor stability." See Office Action, pg. 5. The protective nontoxic proteins are carrier proteins, and co-purify with the toxin chains to form complexes of similar size (900 kD) to the Schantz and Johnson stable Botulinum toxin A crystalline form. Compare Schantz & Johnson, pg. 44, last full paragraph to pg. 9, lines 2-4 of the instant application. Therefore, the Examiner's contention that there is no support for the stability of botulinum toxins other than type B is incorrect. The Examiner cannot compare evidence from other experiments that are not reflective of the conditions put forth in the instant application.

Applicants believe that they have provided extensive teaching regarding the purification of toxin, and preparing it in a formulation that is stable for extended periods of time. Purification of types A and B are described in detail in section III of the specification beginning at about page 10, line 22. As noted at page 12, line 22, toxin types C_1, C_2, D, E, F or G may be prepared and purified according to methods known in the art. One skilled in the art would understand how to adapt the disclosed methods to provide stable formulations comprising types A and C-G from reading the specification and references available prior to the filing of this application.

Based on the amendment to the claims and the remarks above, Applicants respectfully request withdrawal of this rejection.

II. Claim Rejections Under 35 U.S.C. 102(b) to Schantz et al.:

The Examiner rejected claims 16, 17, 21 and 27 under 35 U.S.C. 102(b) as allegedly anticipated by Schantz et al. (J. AOAC 61:96-99 (1978). It is believed that this rejection is not applicable in view of the present amendments to the claims.

The Examiner states that Schantz teaches a solvent composition in buffer having a pH of 4.2. See Office Action, p. 10. Applicants respectfully submit that Schantz does not disclose, teach or suggest a composition as provided in the amended claims. Schantz, therefore, does not anticipate the presently claimed compositions.

In light of the arguments presented above and the amended claims, Applicants respectfully request that the Examiner withdraw the rejection.

CONCLUSION

In light of the remarks and amendments set forth above, Applicant believes that the claims are in condition for allowance. Applicant respectfully solicits the Examiner to expedite the prosecution of this patent application to issuance. Should the Examiner have any questions, the Examiner is encouraged to telephone the undersigned.

Respectfully submitted,

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MICROBIOLOGICAL METHODS

Standardized Assay for *Clostridium botulinum* Toxins

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To improve the accuracy and precision of the mouse assay for botulinum toxins found in foods and body fluids, a stable reference standard of botulinum toxin has been developed. It contains 100 ng crystalline type A botulinum toxin/ml 0.05*M* pH 4.2 sodium acetate buffer which contains 3 mg bovine serum albumin and 2 mg gelatin/ml. The albumin and gelatin stabilize the toxin. For distribution to laboratories carrying out the assay, 0.5 ml of the standard is sealed in 1 ml glass vials. The standard reference toxin, used as directed, enables the assayer to establish the response of laboratory mice, under a specific set of conditions, to a definite amount of toxin and to express the toxin content of an unknown in terms of ng toxin/g or ml food or body fluid. The mouse assay by itself is not specific for botulinum toxin, but when used in conjunction with specific antisera for identification and typing, it is the most reliable test available.

The mouse assay for *Clostridium botulinum* toxins found in foods and human and animal body fluids, when used in conjunction with specific antisera for identification and typing, is the only test for these toxins approved by the Food and Drug Administration (FDA). Mouse assays for the quantitative determination of botulinum toxin from various laboratories have shown a wide range of results. Differences of 4- and 5-fold are common and differences of as much as 10-fold have been reported (1). The reasons for such variations seem to be due mainly to differences in strains of mice used, conditions under which the mice are housed, and techniques used by the assayers. In an attempt to obtain more accurate and precise results from the assay, a reference standard for type A botulinum toxin has been developed and a standardized assay procedure for its use is proposed in this paper.

The purpose of the reference standard is to provide to the assayer a solution containing a definite concentration of toxin. It should give the assayer, particularly if he is inexperienced, an opportunity to observe the signs of botulism in mice and distinguish between deaths due to botulism and nonspecific conditions such as peritonitis and to determine the response of the assayer's animals, under the conditions in which they are used, to a definite amount of the toxin. An unknown sample of food, blood, or other substances containing toxin is compared with the reference standard and the toxin content of the unknown is expressed in ng type A toxin/unit weight or volume. Although signs of botulism in mice challenged with food or other extracts are a good indication that botulinum toxin is present, the mouse assay by itself is not specific for these toxins and specific antisera for the individual types must be employed to positively identify and type the toxins. In this respect, the reference standard type A should also give an inexperienced assayer an opportunity to determine if the neutralization with antiserum to type A toxin is carried out properly.

Because all types of botulinum toxin appear to have a similar mode of action in causing botulism by a presynaptic block, it should be allowable to express the concentration of types other than A in terms of type A equivalents. Although the types of toxin other than A may be expressed as type A equivalents, it is imperative that the type be specifically identified since treatment for victims of botulism is contingent on the type present.

METHOD

Reagents and Materials

(a) *Reference standard toxin.*—Prepare with crystalline (3×) type A (Hall strain) toxin in solvent consisting of 0.05*M* pH 4.2 sodium acetate buffer containing 3 mg bovine serum albumin and

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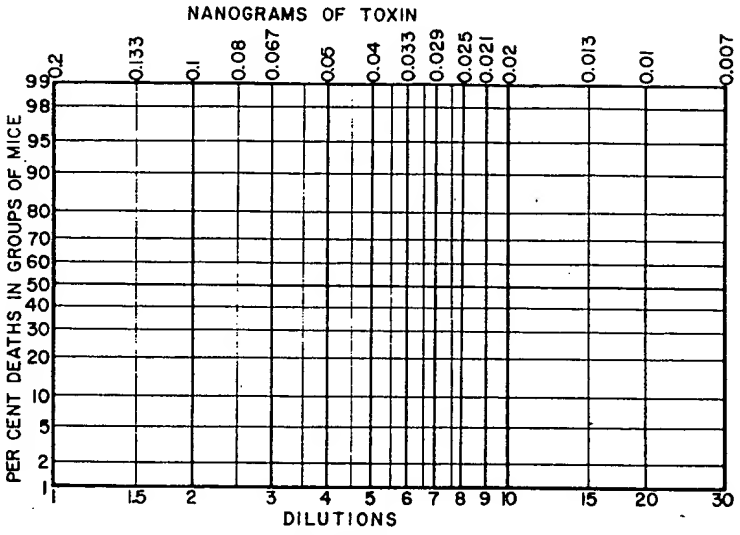

FIG. 1—Chart for plotting dose of toxin in ng or comparable dilutions against per cent deaths in individual groups of mice. Dose at point where the best straight line drawn through points intersects 50% line is taken as LD_{50} and ng toxin for this LD_{50} is read from the upper axis. Dilutions for calculation of toxin in an unknown are read from the lower axis.

2 mg gelatin/ml. The toxin concentration is 100 ng/ml. It is precisely measured by the absorbance at 278 nm (extinction coefficient 1.65 for 1 mg/ml in 1 cm light path) of a solution of toxin containing 2–5 mg toxin and diluted to 100 ng/ml. This standard could be dispensed by the FDA in 1 ml sealed glass vials each containing 0.5 ml (50 ng toxin) to laboratories requesting its use. The proteins added to the solution stabilize the toxin at this pH so that there is no appreciable loss in toxicity for at least 2 years. The solution should be stored at room temperature (preferably 18–24°C), but must not be frozen or the toxicity will be destroyed.

(b) *Mice.*—White males and females weighing between 18 and 22 g and housed in clean, temperature-controlled rooms with ample food and water.

(c) *Sterile sodium phosphate buffer.*—0.05*M*, pH 6.2, containing 0.2% gelatin; use for all serial dilutions.

(d) *Log-probability chart.*—See Fig. 1.

Procedure

Break vial containing reference standard at blue line, remove 0.2 ml, place in flask, and dilute to 30 ml with phosphate buffer. This is the base solution for further use and dilution. It contains 0.4 ng toxin/ml and should be used for assay as soon as possible after preparation. In all cases, the challenge dose to a mouse is 0.5 ml, given intraperitoneally (IP).

For broad range test, give IP dose of 0.5 ml (0.2 ng toxin) base solution to each of 2 or 3 mice and 0.5 ml dose (0.02 ng toxin) of 1:10 dilution to another group of 2 or 3 mice. All mice on base solution (0.2 ng dose) should die unless the mice are very resistant, and all mice on 1:10 dilution (0.02 ng) should survive unless the mice are very susceptible to toxin. The main value of this broad range test is to get a rough measurement of the response of the assay animals to the toxin, which usually can be made within 24 hr. Results of this broad range test should help determine the range of dilutions of the base solutions for a more detailed assay. It is suggested, however, that the base solution be used to make 1.5-, 2.5-, 4-, 6-, 10-, and 15-fold dilutions covering doses of 0.133–0.013 ng in 0.5 ml. Generally, 6 or more mice are used for each dilution. Observe the mice at least twice each day over a 3-day period (72 hr) and record the number dead at each dilution.

Calculation

Plot per cent of mice dead at each dilution on chart (Fig. 1), and draw best straight line by inspection through points between highest dilution where all mice are dead and lowest dilution where no mice are dead. Read ng toxin equivalent to 1 mouse LD_{50} from x-axis at top of chart at point corresponding to where plotted line crosses 50%

This paper was presented at the 90th Annual Meeting of the AOAC, Oct. 18–21, 1976, at Washington, DC.

line. This figure is the ng factor for the mice and conditions under which the assay was carried out and is used in the calculation of the ng toxin in an unknown sample.

Assay of Unknown Samples

An unknown sample is assayed in the same manner as the standard toxin. Prepare food or other substance to be assayed and extract toxin as directed in the *Bacteriological Analytical Manual for Foods* (2). Obtain range of concentration of toxin by making 10-fold dilutions of extract or solution to be assayed. If, for example, test mice die at all dilutions up through 10^4 and none die at 10^5 dilution, then further dilute 10^4 dilution in a manner similar to reference standard and use 6 mice on each dilution. Observe mice at times over 72 hr period and record number dead in each group. Plot per cent of mice dead at each dilution on the log-probability chart and determine LD_{50} in the same manner as with the reference standard. In this particular case however, read number of dilutions equivalent to one LD_{50} from bottom axis of chart. This value $\times$ prior dilution of extract $\times$ 2(mouse dose 0.5 ml) = number of mouse LD_{50}/ml extract. $LD_{50} \times$ ng factor (which is ng toxin equivalent to 1 mouse LD_{50}) = ng toxin/ml extract.

Interlaboratory Study

The standard toxin, along with directions for its use as described in this paper, was distributed to various laboratories by the Division of Microbiology of the FDA for test and determination of the ng factor. Results from 11 laboratories were: 0.045, 0.036, 0.075, 0.045, 0.033, 0.051, 0.045, 0.032, 0.040, 0.039, and 0.036 ng toxin equivalent to 1 mouse LD_{50}. The average was 0.043; the standard deviation was 0.012. Because the assays were carried out according to a definite procedure used by all laboratories, the results reflect for the most part the variation in the response of the mice to the toxin in each laboratory.

Discussion

A reference standard is necessary for good analytical procedures. Highly purified botulinum toxin is an unstable protein (3), particularly in the dilute solution used in the reference standard. We have, however, used a solvent in which the toxin maintains toxicity at room temperature for at least 2 years without significant loss. The crystalline toxin at a concentration of ≥ 2 mg/ml pH 4.2 acetate buffer maintains its toxicity over long periods, but when diluted to <1 mg/ml, the toxicity decreases rapidly. At 100 ng/ml, the concentration used in the reference standard, the toxicity is almost entirely lost within a few days unless another protein is added to the solution. In this case we used bovine serum albumin and gelatin to maintain stability.

The reference standard is packaged in quantities of 50 ng for safety reasons. This amount is considered well below the dose to cause botulism in a human but sufficient to carry out tests on over 1000 mice. One disadvantage to dissolving the toxin in pH 4.2 acetate buffer is that it cannot be frozen without loss of toxicity. Even refrigeration at 4°C is not advisable unless the toxin solution is allowed to stand at room temperature for several hours before being assayed. The reason for this phenomenon is not understood, but we have some evidence that the shape of the molecule is distorted to a nontoxic form when the solution is frozen in acetate buffer. This is not the case when the toxin is dissolved in pH 6.2 sodium phosphate buffer. In this buffer the solution can be frozen and thawed without appreciable loss of toxicity. However, the toxicity in phosphate buffer at room temperature is not maintained more than a few days without considerable loss.

Type A botulinum toxin is the only one of the 7 recognized types that is available in crystalline form. Our experience with the crystalline toxin shows that it is uniform in chemical and physical properties from one preparation to another and uniform in toxicity when assayed with a single strain of mice under uniform conditions. Because the toxicity varies in different strains of mice and the conditions under which they are kept, the reference standard should be made with crystalline toxin and the concentration should be based on its chemical and physical properties. For this purpose we used the extinction coefficient of 1.65 for 1 mg toxin/ml in a light path of 1 cm at 278 nm and a 260/278 nm absorbance ratio of ≤ 0.55 to quantitatively measure the toxin. To prevent loss of toxicity during the preparation of the standard solution, the absorbance was measured on a toxin solution at a concentration of 2–5 mg/ml and diluted to 100 ng/ml with the acetate buffer containing the serum albumin and gelatin.

Funds for these studies were furnished under Contract No. 223-74-2157 of the FDA and the Food Research Institute, College of Agricultural and Life Sciences, University of Wisconsin-Madison.

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gelatin.

Some question might be raised regarding the use of type A toxin as a standard for the other types, but on the basis of the following information we feel justified in using the most suitable type as the standard for all of the types. Types other than A have not been obtained in crystalline form and purified preparations of these types have not proved sufficiently uniform in composition to serve as a standard. As far as we know, all types of botulinum toxins are simple proteins that have the same or very similar modes of action, resulting in paralysis by a presynaptic block of nerve transmission.

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